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Agrobacterium rhizogenes Mutants That Fail To Bind to Plant Cells

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Transposon insertion mutants of *Agrobacterium rhizogenes* were screened to obtain mutant bacteria that failed to bind to carrot suspension culture cells. A light microscope binding assay was used. The bacterial isolates that were reduced in binding to carrot cells were all avirulent on *Bryophyllum diagremontiana* leaves and on carrot root disks. The mutants did not appear to be altered in cellulose production. The composition of the medium affected the ability of the parent and mutant bacteria to bind to carrot cells. The parent strain bound to carrot cells in greatest numbers in low-ionic-strength media such as 4% sucrose but still showed significant binding in Murashige-Skoog tissue culture medium. All of the mutants showed reduced binding in 4% sucrose after 2 h of incubation with carrot cells. One mutant was delayed in binding in 4% sucrose. This mutant and one other mutant also showed reduced binding to carrot cells in Murashige-Skoog medium. To determine whether the Tn*5* insertion was responsible for the mutant phenotype, DNA containing the Tn*5* insertion was cloned from the mutant bacteria and used to introduce Tn*5* into the parent strain in the same location as in the original mutant by marker exchange. The resulting transconjugants had the same avirulent, nonattaching phenotype as the original mutants, suggesting that the mutant phenotype was due to the Tn*5* insertion. The cloned DNA containing the Tn*5* insertion was also tested for homology to DNA of known genes that affect attachment of *Agrobacterium tumefaciens* to plant cells by DNA hybridization. No homology to *chv*, *att*, or *pscA* clones was observed. In addition, cloned *chv*, *att*, and *pscA* genes from *A. tumefaciens* were unable to complement the attachment-minus *A. rhizogenes* mutants. Thus, the *A. rhizogenes* nonattaching mutants appear to be different from the previously described *A. tumefaciens* mutants.

One of the early events in tumor formation by *Agrobacterium tumefaciens* is attachment of the bacteria to the host cell surface (6). Bacterial attachment appears to be required for tumor formation; all nonattaching mutants that have been isolated are avirulent (2, 9, 17).

Agrobacterium rhizogenes (the causative agent of hairy root disease) has the same basic pathogenic mechanism as *A. tumefaciens*: the transfer of plasmid DNA from the bacterium to the host plant. However, attachment of *A. rhizogenes* to host cells appears to be reduced compared with that of *A. tumefaciens* biotype 1 (16). Unlike the case for *A. tumefaciens*, attachment of *A. rhizogenes* is sensitive to the ionic strength of the medium (16). To determine whether attachment of *A. rhizogenes* to host cells is required for hairy root formation, nonattaching mutants of *A. rhizogenes* were isolated and characterized.

MATERIALS AND METHODS

Bacterial strains and culture media. *A. rhizogenes* A4PC was obtained from Mary-Dell Chilton, CIBA-GEIGY, Research Triangle Park, N.C. The sources of *Escherichia coli* 1830(pJB4JI), JM83, GM4(pBR322), and HB101(pRK2013) were as previously described (8–10). *E. coli* HB101 containing the cosmid clone pCD523, which contains the *chvAB* genes (3), and pJ2.0, which contains the *pscA* gene (17), were obtained from Gerard Cangelosi and Michael Thomashow, respectively. *E. coli* Dh5α containing the cosmid clone pCP13.108.2, which contains the *att* genes (9), was isolated from a library of *A. tumefaciens* NT1 DNA obtained from Stephen Farrand (A. G. Matthysse, unpublished data). All of these clones contain wild-type DNA and complement the attachment defect of the particular mutant used to obtain them. *A. tumefaciens* wild-type strain C58, its avirulent derivative NT1, and the nonattaching mutant AttC-43 derived from strain C58 were as previously described (9). *A. rhizogenes* was grown in nutrient broth or on nutrient agar (Difco Laboratories) plates at 25°C. The minimal medium used for *A. rhizogenes* was that of Roberts and Kerr (14). *E. coli* strains were grown in Luria broth at 37°C. *A. tumefaciens* strains were grown in Luria broth at 25°C. Bacteria used for attachment assays were grown to early stationary phase in Luria broth or nutrient broth. Antibiotics were added at the following concentrations: neomycin, 60 μg/ml; gentamycin, 50 μg/ml; and tetracycline, 5 μg/ml.

Transposon mutagenesis and isolation of mutants. The transposon Tn*5* was introduced into *A. rhizogenes* A4PC by conjugation from *E. coli* 1830(pJB4JI) as previously described (8). *A. rhizogenes* transconjugants containing Tn*5* were selected on Roberts-Kerr minimal medium (14) containing 60 μg of neomycin per ml. Neither parent strain grew under these conditions (*E. coli* 1830 is a multiple auxotroph). As well as carrying Tn*5*, pJB4JI carries a gene for gentamicin resistance. Transconjugants were tested on gentamicin-containing medium to confirm that pJB4JI was not maintained in them. Approximately 325 transconjugants were screened for the ability to attach to carrot cells, using a microscopic attachment assay as previously described (11). Five mutants with altered attachment properties were obtained. The mutation rate to an attachment-minus phenotype therefore was approximately 1.5%, similar to the rate at which nonattaching mutants are recovered for *A. tumefaciens* by using this protocol (about 0.5%; 9).

Bacterial mutants that bound to carrot cells but were

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unable to aggregate the plant cells were also observed during the screening of the mutant bacteria.

Attachment assays. *Daucus carota* was grown in liquid suspension culture in Murashige-Skoog (MS; 12) medium on a shaker at 28°C. Early-stationary-phase cells were diluted to 2×10^5 to 5×10^5 cells per ml, and 2×10^6 bacteria per ml were added. Samples of the incubation mixture were observed under the light microscope after 2, 4, and 24 h and were scored for bacterial attachment to the plant cells and for plant cell aggregation. Bacterial attachment to carrot cells was photographed by using living material and a Zeiss photoscope 2 with Nomarski optics. Samples were prepared for scanning electron microscopy as previously described (10).

The time course of bacterial attachment to carrot cells was examined as previously described (11). Briefly, 2×10^3 to 3×10^3 bacteria per ml were incubated with 2×10^5 to 5×10^5 carrot cells per ml in 4% sucrose or in MS medium. At this ratio of bacteria to carrot cells, carrot cells are present in vast excess. Samples were taken after various incubation times, and the free bacteria were separated from the carrot cells and attached bacteria by filtration through Miracloth (Calbiochem) filters. The numbers of free and attached bacteria were determined by viable cell counts as previously described (11).

Characterization of the mutants. To determine whether the bacteria were altered in the synthesis of extracellular β-linked polysaccharides, including cellulose, bacteria were plated on nutrient agar containing cellufluor (2.5 μg/ml), which gives a fluorescent stain with β-linked polysaccharides (4, 8). No difference in staining of the parent and mutant bacteria was seen, suggesting that the mutants were not altered in cellulose synthesis.

Bacterial virulence was tested on *Bryophyllum diagremontiana* by using toothpick wounds and on carrot disks by using procedures previously described (8, 9). *B. diagremontiana* was grown in the greenhouse. Carrots were purchased at a local store.

DNA isolation, hybridization, and cloning. DNA was extracted from *A. rhizogenes* as previously described for *A. tumefaciens* (8). The determination of whether Tn*5* was located in the bacterial chromosome or in a plasmid was carried out as previously described (8). Tn*5* was located in the bacterial chromosome in all of the nonattaching mutants. The Tn*5* mutants were tested for the presence of Mu DNA (resulting from the presence of Mu in pJB4JI) by DNA hybridization as previously described (8). Mu DNA was detected in one of the nonattaching mutants that was also unstable. This mutant was not characterized further. No Mu DNA was detected in the other four nonattaching mutants. Bacterial DNA was prepared from mutant *A. rhizogenes* and digested with *Eco*RI; the fragment carrying Tn*5* was identified by hybridization to Southern transfers of DNA fragments separated by agarose gel electrophoresis as previously described (8). *A. rhizogenes* DNA from the nonattaching mutants was digested with *Eco*RI and cloned into the *Eco*RI site of pBR322 as described by Maniatis et al. (7). The resulting plasmids were transformed into *E. coli* JM83, and the transformants were selected for neomycin resistance and subsequently for tetracycline resistance.

To determine whether the Tn*5* insertion was responsible for the nonattaching phenotype, the cloned *A. rhizogenes* DNA containing the Tn*5* insertion was introduced into the wild-type parent strain by marker exchange (15). A triparental mating was carried out, using *E. coli*(pRK2013) to mobilize the transfer of the pBR322 clones from *E. coli* to *A. rhizogenes*. Cultures of *E. coli* JM83 carrying the plasmid with the cloned DNA fragment and *E. coli* HB101(pRK2013) were grown to mid-log phase at 37°C in Luria broth. *A. rhizogenes* was grown to mid-log phase in nutrient broth at 25°C. Volumes of the bacterial cultures containing approximately equal numbers of bacteria were mixed on nutrient agar plates and incubated overnight at 25°C to allow mating to occur. Bacteria were harvested by scraping the plates and were washed once with 0.09% NaCl. Transconjugants were selected for the ability to grow on minimal medium containing 60 μg of neomycin per ml. None of the parent strains grew under these conditions, nor did transconjugants from matings involving any two parent strains without the third strain. Since plasmid pBR322 cannot be maintained in *A. rhizogenes*, neomycin-resistant transconjugants result from the integration of all or part of the plasmid carrying the cloned *A. rhizogenes* DNA with the Tn*5* insertion. Transconjugants were tested for the retention (presumably from integration into the bacterial chromosome by a single homologous recombination event) of the entire pBR322 by testing for tetracycline resistance. At least two isolates from each cross that were and that were not resistant to tetracycline were tested for the ability to bind to carrot cells in the microscopic attachment assay. Isolates that appeared to contain all of pBR322 (and thus would be expected to contain a wild-type and a mutant copy of the attachment genes) were able to bind to carrot cells. Isolates that were neomycin resistant and tetracycline sensitive and did not appear to contain pBR322 (which presumably arose by a double homologous recombination event and would be expected to contain only the mutant copy of the attachment genes) were unable to attach to carrot cells. The properties of the transconjugants were characterized further as described for the original mutants.

Hybridization to and complementation by cloned *A. tumefaciens* genes. The cloned DNA from the *A. rhizogenes* mutants containing the Tn*5* insertion was digested with *Eco*RI, vector DNA was separated from the insert by electrophoresis in 0.7% agarose, and Southern transfers were prepared and hybridized to plasmids pCD523, pJ2.0, and pCP13.108.2 labeled with ^{32}P by using a random primer kit (Amersham) as instructed by the manufacturer. Hybridization was carried out in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C, and the filters were washed in 4× SSC–0.1% sodium dodecyl sulfate at 40°C. These low-stringency conditions were used to increase hybridization between *A. rhizogenes* and *A. tumefaciens* chromosomal DNA. The hybridization experiment was also carried out as described above by using Southern transfers of *Eco*RI-digested DNA from the *A. tumefaciens* clones pCD523, pJ2.0, and pCP13.108.2 separated by gel electrophoresis and hybridized to radioactive cloned DNA from each of the *A. rhizogenes* mutants.

To determine whether the *A. tumefaciens* wild-type DNA clones pCD523, pJ2.0, and pCP13.108.2 could complement the *A. rhizogenes* nonattaching mutants, these plasmids were introduced into the mutants by conjugation from *E. coli*, using *E. coli* HB101(pRK2013) to mobilize the plasmids for conjugation. The plasmids containing the clones are all derivatives of pRK290 and can be maintained in *A. rhizogenes*. Transconjugants were selected for tetracycline resistance carried on the plasmid and for growth on minimal medium. The ability of the transconjugants to attach to carrot cells was assayed as described above except that tetracycline (5 μg/ml) was added to the medium to prevent growth of *A. rhizogenes* that had not retained the plasmid.

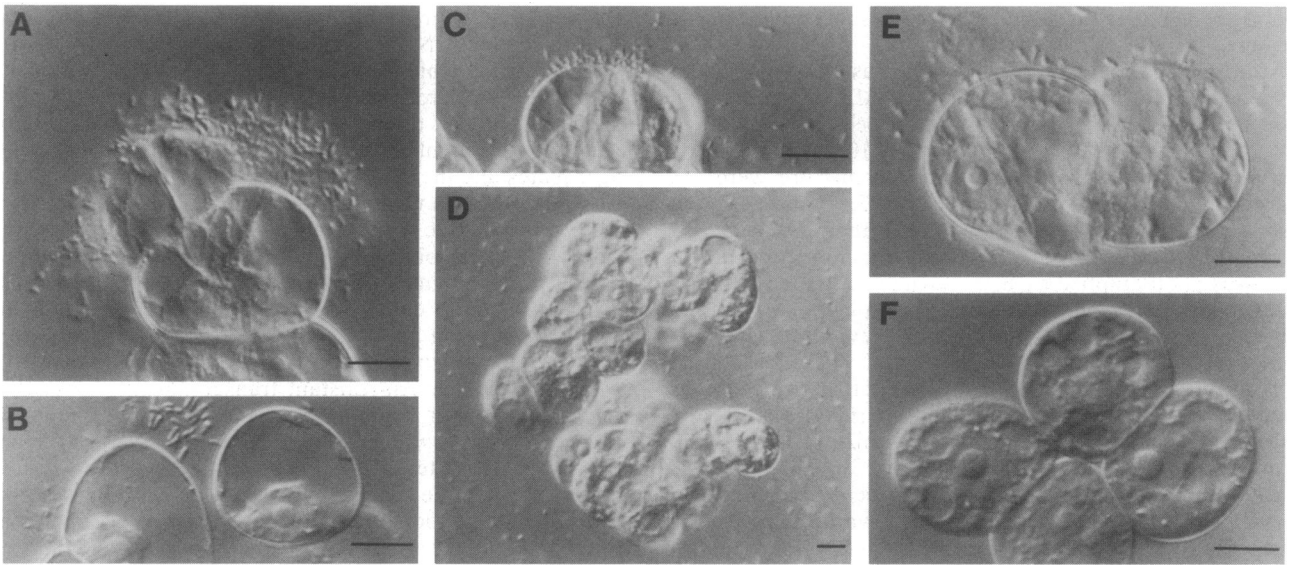

FIG. 1. Photomicrographs using Nomarski optics of *A. rhizogenes* strains incubated for 24 h with carrot suspension culture cells. (A) Strain A4PC incubated with carrot cells in 4% sucrose; (B) strain A4PC incubated with carrot cells in MS medium; (C) mutant Att-323 incubated with carrot cells in 4% sucrose; (D) mutant Att-323 incubated with carrot cells in MS medium; (E) mutant Att-325 incubated with carrot cells in 4% sucrose; (F) mutant Att-325 incubated with carrot cells in MS medium. Attachment of the parent strain A4PC was reduced in MS medium compared with that of 4% sucrose. Attachment of both mutants appeared to be reduced but detectable in 4% sucrose. No attachment of either mutant in MS medium was observed.

RESULTS

Characterization of the attachment of *A. rhizogenes* to carrot suspension culture cells. Attachment of *A. rhizogenes* A4PC to carrot suspension cells was previously shown to be sensitive to the ionic strength of the medium (16). Bacterial attachment was therefore examined both in 4% sucrose and in MS medium with ionic strengths of less than 0.01 and 0.09 M, respectively. The bacteria showed only a very small percentage of the inoculum attached to carrot cells after 2 h in MS medium. After 24 h of incubation with carrot cells in MS medium, attached bacteria were visible in the light microscope (Fig. 1).

A larger percentage of the bacterial inoculum attached to carrot cells in 4% sucrose than in MS medium. Bacterial attachment was visible in the light microscope after 4 h of incubation with carrot cells in 4% sucrose; the number of visible attached bacteria increased significantly with longer periods of incubation (Table 1). When carrot cells with attached bacteria were examined with the scanning electron microscope, more attached bacteria were seen with carrot cells incubated in 4% sucrose than with carrot cells incubated in MS medium. Fibrils were observed surrounding the attached bacteria. Similar fibrils were also produced by bacteria grown in nutrient broth with 0.02% Soytone (Fig. 2). Fibrils with a similar appearance have been observed surrounding *A. tumefaciens* attached to carrot cells. In the case of *A. tumefaciens*, these fibrils have been shown to be composed of cellulose synthesized by the bacteria (10). The fibrils produced by *A. rhizogenes* were observed to fluoresce with cellufluor stain in the light microscope. Cellufluor stains β-linked polysaccharides, including cellulose (4). The fibrils could be removed by treatment of the bacteria bound to carrot cells with cellulase (data not shown). Thus, it appears likely that the fibrils observed surrounding *A. rhizogenes* are composed of cellulose.

The attachment of *A. rhizogenes* to carrot cells was compared with that of wild-type *A. tumefaciens* biotype 1 and of a nonattaching mutant of *A. tumefaciens* (Table 1). After 2 h of incubation in MS medium, biotype 1 *A. tumefaciens* C58 cells were observed attached to carrot cells. Viable cell count measurements showed 40% of the bacterial inoculum bound to the carrot cells. Neither *A. rhizogenes* nor the nonattaching *A. tumefaciens* mutant Att-C43 showed any detectable attachment in the light microscope after 2 h of incubation in MS medium. Only 3% (or less) of the bacterial inoculum was bound to the plant cells. In 4% sucrose, the binding of *A. rhizogenes* A4PC to carrot cells was increased. Attached bacteria could be seen in the light microscope; 19% of the bacterial inoculum bound to the carrot cells. Incubation in 4% sucrose had little or no effect on the binding of the *A. tumefaciens* nonattaching mutant Att-C43. Thus, the binding of wild-type *A. rhizogenes* to carrot cells appeared to be distinct from both that of wild-type *A. tumefaciens* and that of the nonattaching mutant of *A. tumefaciens*.

Characterization of nonattaching mutants of *A. rhizogenes*. To determine the role of bacterial attachment in the virulence of *A. rhizogenes* and to examine the relationship between attachment in 4% sucrose and in MS medium, transposon mutants of *A. rhizogenes* that failed to bind to carrot cells were examined. These mutants were isolated by screening transposon mutants for those which showed reduced binding to carrot cells after 24 h of incubation in 4% sucrose or in MS medium. Four stable mutants were obtained and characterized. All of the mutants showed reduced binding to carrot cells after 2 h of incubation in 4% sucrose; 4% or less of the mutant bacterial inoculum bound, compared with 19% of the parent strain (Fig. 1 and Table 1). All of the mutants also showed reduced binding in MS medium, as judged by examination in the light microscope after 24 h of incubation (Fig. 1). It was difficult to obtain reliable measurements of the percentage of the bacterial inoculum bound in 2 h in MS medium for either the parent strain or the mutants since the percentage was small even for the parent

TABLE 1. Characteristics of nonattaching bacterial mutants

Bacterial strain	Attachment to carrot cells in:					Ability to aggregate carrot cells in 4% sucrose	Virulence on:	
	4% Sucrose			MS[a]				
	Microscopic[b] 4 h	Microscopic[b] 24 h	% of inoculum attached[c]	Microscopic, 24 h	% of inoculum attached		*B. diagremontiana*	Carrot
A. tumefaciens								
C58 (wild type)	+	++	35 ± 4	+	40 ± 5	+	+	+
Att-C43	−	−	5 ± 7	−	3 ± 4	−	−	−
A. rhizogenes								
A4PC (parent, wild type)	+	++	19 ± 6	+	2.5 ± 0.9	+	+	+
Mutants								
Att-310	−	+	4 ± 3	−	0.4 ± 0.1	−	−	−
Att-312	−	+	3 ± 3	−	1.0 ± 0.1	−	−	−
Att-323	+	+	3 ± 4	−	1.2 ± 0.6	−	−	−
Att-325	+	+	3 ± 4[d]	−	0.6 ± 0.2	+	−	−
Transconjugants[e]								
Att-310-1	+	+	2 ± 2	−	0.4 ± 0.2	−	−	NT[f]
Att-312-1	−	+	4 ± 4	−	1.7 ± 0.5	−	−	NT
Att-312-2	−	+	3 ± 3	−	0.4 ± 0.1	−	−	NT
Att-325-2	+	+	1 ± 2[d]	−	0.6 ± 0.3	+	−	NT

[a] Containing 4% sucrose.
[b] 10^7 bacteria per ml incubated with 10^5 carrot cells per ml. −, No detectable attachment; +, 1 to 10 bacteria attached per carrot cell clump; ++, 10 to 100 attached bacteria per carrot cell clump.
[c] Bacteria (2×10^3 to 3×10^3/ml) incubated with 10^5 carrot cells per ml for 2 h. Values are means and standard deviations of at least three separate experiments.
[d] At 4 h, Att-325 and Att-325-2 showed 14 ± 7% of the bacterial inoculum attached. None of the other mutants showed increased attachment with incubation times longer than 2 h, nor did Att-325 show increased attachment at 4 h in MS medium.
[e] Tetracycline-sensitive, Tn*5*-containing *A. rhizogenes* strains that arose from a double homologous recombination event involving plasmid pBR322 containing the mutated attachment locus.
[f] NT, Not tested.

strain. However, two of the mutants, Att-310 and Att-325, appeared to be reduced in binding in MS medium compared with the parent strain. No difference was seen between the binding of the parent strain and of a random Tn*5*-containing mutant of the parent strain.

When wild-type *A. rhizogenes* cells were incubated with carrot suspension culture cells in 4% sucrose, the bacteria that bound to the plant cells and the associated bacterial fibrils caused the formation of large clusters of bacteria on the plant cell surface and the aggregation of the carrot cells. All of the nonattaching mutants showed normal fluorescence on nutrient agar plates containing cellufluor and normal production of fibrillar material which fluoresced with cellufluor stain in the fluorescent light microscope, suggesting that they were unaltered in their ability to produce cellulose. Three of the nonattaching mutants failed to aggregate carrot cells in 4% sucrose. The failure of nonattaching mutants to aggregate plant cells is presumably due to the lack of bacterial binding to the plant cell surface. Nonattaching mutants of *A. tumefaciens* C58 also fail to aggregate carrot cells (9). However, one of the nonattaching mutants of *A. rhizogenes*, Att-325, did aggregate carrot cells in 4% sucrose. Further examination of the binding of this mutant to plant cells showed that it was delayed in binding in 4% sucrose. Although only 3% of the bacterial inoculum was bound after 2 h, after 4 h 14% of the bacteria were bound. (After 4 h, 25% ± 6% of the parent bacteria were bound to the carrot cells.) None of the other mutants showed increased binding after 4 h of incubation in 4% sucrose or in MS medium. Att-325 also did not show increased binding with increased incubation time in MS medium.

The virulence of the nonattaching mutants was determined on leaves of *B. diagremontiana* and on carrot disks. The parent bacteria were virulent and induced the formation of hairy roots on *Bryophyllum* leaves and carrot disks. The mutants were avirulent on both plants (Fig. 3 and 4).

The site of the Tn*5* insertion in the nonattaching mutants was examined by lysing the bacteria in the well and separating the Ri plasmid DNA from the chromosomal DNA by electrophoresis. Radioactive Tn*5* DNA was hybridized to a Southern transfer of this gel, and autoradiography was performed. The Tn*5* DNA showed hybridization only to the broad band of chromosomal DNA and not to the plasmid DNA, suggesting that the Tn*5* insertions in the mutants were chromosomal (data not shown).

To determine whether the Tn*5* insertions in the four mutants were located near each other in the bacterial chromosome, DNA from the nonattaching mutants was digested with *Eco*RI and separated by agarose gel electrophoresis (Tn*5* lacks *Eco*RI sites). Radioactive Tn*5* DNA was hybridized to Southern transfers of this DNA. The Tn*5* DNA hybridized to a single band of 13 to 14 kb in each of the nonattaching mutants. The *Eco*RI DNA fragment containing the Tn*5* insertion from the nonattaching mutants was cloned into the *Eco*RI site in pBR322. The cloned *A. rhizogenes* DNA was digested with *Eco*RI and *Hin*dIII, and the fragments were separated by gel electrophoresis and transferred to nitrocellulose (four identical filters were prepared). There appeared to be at least three *Hin*dIII sites in the cloned DNA in addition to the two *Hin*dIII sites present in Tn*5*. Each filter was hybridized to radioactive DNA from one of the four cloned mutant DNA plasmids prepared by using a random primer kit (Amersham). All of the bands from the other mutant clones on the filters hybridized to each radioactive DNA probe, suggesting that the Tn*5* insertion in each of the mutants was in the same *Eco*RI fragment of the bacterial chromosome (data not shown).

To determine whether the nonattaching and avirulent

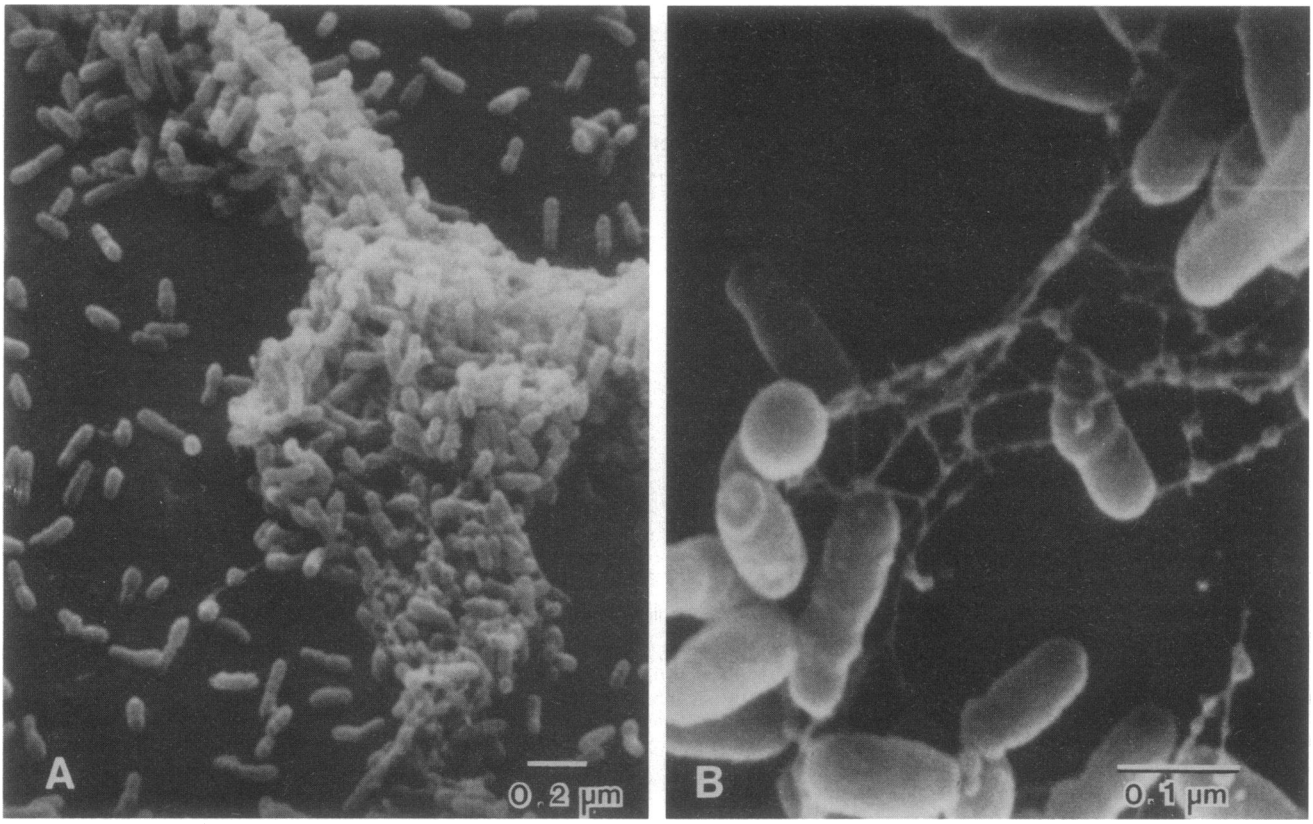

FIG. 2. Scanning electron micrographs of *A. rhizogenes* grown in nutrient broth with 0.02% Soytone, showing fibril production by the bacteria. (A) Clumped bacteria; (B) higher-magnification view of bacteria from the edge of a clump.

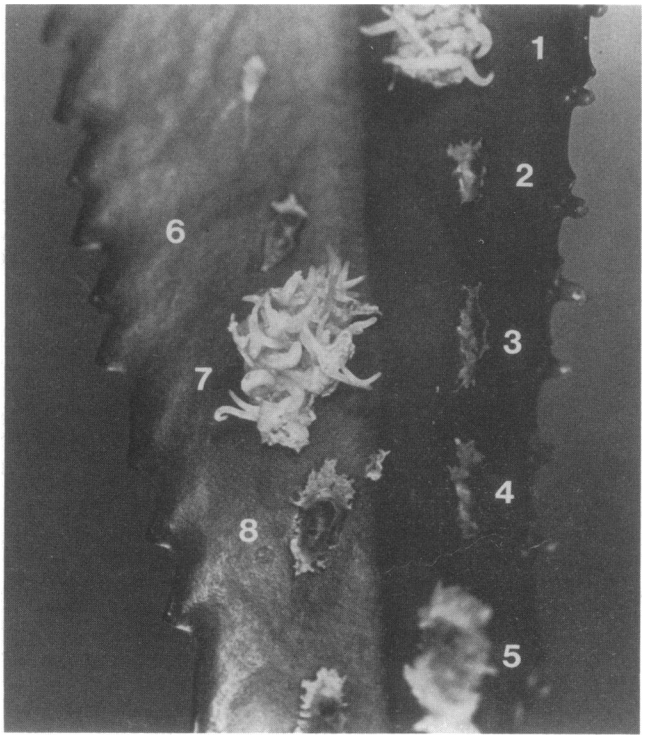

FIG. 3. Leaf of *B. diagremontiana* inoculated with *A. rhizogenes* at the numbered sites and allowed to grow for 6 weeks. The bacteria inoculated were as follows: 1, parent strain A4PC; 2, mutant Att-310; 3, mutant Att-312; 4, mutant Att-323; 5, parent strain A4PC; 6, mutant Att-323; 7, parent strain A4PC; 8, mutant Att-325.

phenotypes resulted from the Tn*5* insertion in the mutants, the *Eco*RI DNA fragment containing the Tn*5* insertion from three of the nonattaching mutations that had been cloned in pBR322 in *E. coli* was used to replace the wild-type DNA in the parent strain A4PC by marker exchange. The resulting transconjugant strains were characterized. They showed the same attachment properties as did the original mutants, including the delayed attachment of Att-325 (Table 1). The transconjugants produced by marker exchange, like the original mutants, were avirulent. These results suggest that the alterations in attachment in 4% sucrose and in MS medium and the lack of virulence were caused by the Tn*5* insertion in the bacterial chromosome.

Relationship between *A. tumefaciens* nonattaching mutants and *A. rhizogenes* nonattaching mutants. Three types of nonattaching mutations of *A. tumefaciens* have been described: *chvAB* (2), *att* (9), and *pscA* (17). Cosmid clones containing the wild-type genes that complement these mutations were introduced into each of the nonattaching *A. rhizogenes* mutants by conjugation. The ability of the transconjugant *A. rhizogenes* to attach to carrot cells was then measured. None of the *A. rhizogenes* nonattaching mutants was complemented to a normal attachment phenotype by any of the three cosmids pCD523, pCP13.108, and pJ2.0.

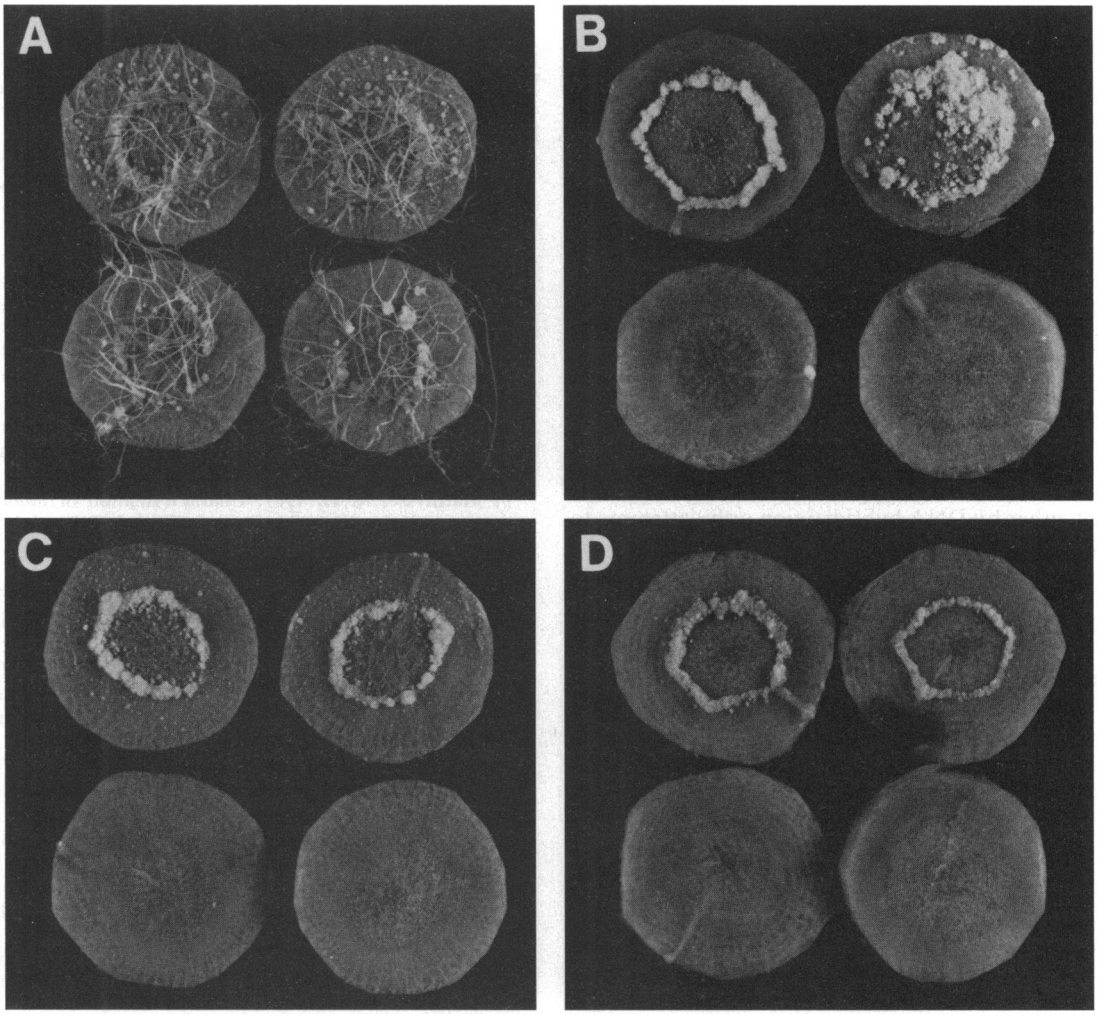

FIG. 4. Carrot disks inoculated with *A. rhizogenes* and allowed to grow for 4 weeks. In each panel, the upper two disks were inoculated with the apical side up and the lower two disks were inoculated with the basal side up. (A) Disks inoculated with the parent strain A4PC; roots formed. (B) Disks inoculated with mutant Att-312; no roots formed. (C) Disks inoculated with mutant Att-325; no roots formed. (D) Disks inoculated with the avirulent strain NT1 of *A. tumefaciens*; the callus formed on the disks incubated with the apical side up appeared to be a wound response.

To check further for a possible relationship between the *A. rhizogenes* nonattaching mutations and known *A. tumefaciens* genes involved in attachment, the cloned *A. rhizogenes* DNA containing the Tn*5* insertions that gave rise to the mutations was hybridized to Southern transfers of *Eco*RI-digested plasmid DNA from plasmids pCD523, pCP13.108, and pJ2.0. No hybridization between the *A. tumefaciens* and the *A. rhizogenes* DNA (except that between the vector DNA bands) was detected. In addition, radioactive pCD523, pCP13.108, and pJ2.0 DNA was hybridized to a Southern transfer of an *Eco*RI digest of the cloned *Eco*RI fragments containing the Tn*5* insertion from *A. rhizogenes* mutants Att-310, Att-312, and Att-325. Again, no hybridization except that with the vector DNA band was detected. Thus, these nonattaching *A. rhizogenes* mutants appear to be distinct from the previously described nonattaching mutants of *A. tumefaciens*.

DISCUSSION

The attachment of *A. tumefaciens* to host cells has been shown to be an early event in tumor formation by the bacteria (6). This attachment is required for virulence; all nonattaching mutants of *A. tumefaciens* isolated thus far are avirulent (2, 9, 17). Attachment of *A. tumefaciens* biotype 1 is not particularly sensitive to the composition of the medium; the percentages of the bacterial inoculum attached are very similar when the bacteria are incubated with carrot cells in 4% sucrose, in MS medium, and in 0.25 M NaCl (11, 13, 16). Nonattaching mutants fail to attach in any of these media (9).

In comparison with *A. tumefaciens* biotype 1, *A. rhizogenes* attaches poorly to carrot suspension culture cells. A smaller percentage of the bacterial inoculum is attached in 4% sucrose. The attachment is sensitive to the ionic strength of the medium; bacterial attachment is much reduced in MS medium and is very slow (16). Although few bacteria bind and the binding is sensitive to ionic strength for *A. rhizogenes*, the mechanism of pathogenicity for *A. rhizogenes* is the same as for *A. tumefaciens*: the transfer of DNA from the bacterial plasmid to the plant host cell, where this bacterial DNA becomes integrated into the host cell chromosomes and its expression causes the formation of tumors or hairy roots (1, 5).

To determine whether the attachment of *A. rhizogenes* was required for bacterial virulence and to examine the question of whether the attachment in 4% sucrose and in MS medium had similar mechanisms, nonattaching mutants of *A. rhizogenes* were obtained. These mutants were avirulent, suggesting that the small amount of bacterial attachment seen with wild-type *A. rhizogenes* is required for DNA transfer from the bacteria to the plant cell. In addition, all of the nonattaching mutants obtained appeared to be reduced in attachment both in 4% sucrose and in MS medium. Thus, it is likely that attachment in the two media involves some of the same genes and gene products. However, there may also be some differences in the requirements for bacterial attachment in 4% sucrose and in MS medium since Att-325, which showed delayed attachment in 4% sucrose, showed reduced but not delayed attachment in MS medium. No relationship between the *A. rhizogenes* nonattaching mutants described in this report and the previously described nonattaching mutants of *A. tumefaciens* biotype 1 (*chvAB*, *att*, and *pscA*) was detected either by DNA hybridization or by attempts to complement the *A. rhizogenes* mutants with the cloned *A. tumefaciens* genes. This result was not unexpected since previous characterization of attachment of *A. tumefaciens* biotype 1 and of *A. rhizogenes* (16) suggested that the mechanism of attachment to host cells may differ between these two groups of bacteria. The mechanism of attachment of *A. rhizogenes* and the nature of the alteration in the bacteria that reduces attachment in these mutants is unknown and is the subject of current investigations.

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